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A BIODEGRADABLE POLYCATION COMPOSITION FOR DELIVERY OF AN ANIONIC MACROMOLECULE

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by:

היום 22 בחודש 07 שנת 1999
This th day of 199

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A BIODEGRADABLE POLYCATION COMPOSITION FOR DELIVERY OF AN ANIONIC MACROMOLECULE

תכשיר פוליקטיון המתקלה-ביולוגית לשילוח מקרומולקולה אניונית

Field of Invention:

The present invention relates to a biodegradable polycation composition for delivery of an anionic macromolecule.

Background:

Gene therapy is a process by which genes are introduced into cells which then become mini-factories which manufacture and release essential compounds in cells and tissue which improve the life of the patient. Gene therapy has the potential to revolutionize the treatment of genetic disorders, diseases associated with a genetic component like cancer, AIDS, and many other diseases. Gene therapy may be the only remedy for some individuals who would otherwise die or be severely disabled. Gene transfer may also be employed for systemic protein and peptide-like hormone administration. Nucleic acid sequences coding for a protein (insulin, growth hormone) would be administered to the patient allowing endogenous production of their own medication.

Successful gene therapy requires the identification of an appropriate therapeutic gene for treatment of the disease, in additioln to a delivery system by which that gene can be delivered to desired cell type both efficiently and accurately. Early attempts of gene transfer involved the removal of cells from the individual, and the alteration of the cells in the culture by the introduction of a functioning copy of the gene. The next step included grafting the genetically engineered cells back into the patient. This ex vivo approach to gene therapy is obviously limited to those target tissues that are not undergoing frequent multiplication and cell generation that could cause progressing elimination of the grafted cells. The ability of the altered cells to efficiently recombine

with the target tissue is another limiting factor of the ex vivo approach since many cells do not exhibit the ability to recombine.

The limitation and the complexity of the ex-vivo approach facilitated the development of direct in vivo gene transfer methods. Direct gene therapy involves the administration of the gene into the body, targeting of the gene to the desired cells and into the nucleus of said genes, and expression of functioning gene products therein. Currently there are two different approaches for direct gene transfer. One is a viral approach and the other is a non-viral approach. Viral and non-viral gene therapies differ in the methods used to deliver genes to the target cells and direct the uptake of gene into the nucleus. Viral gene therapies employ genetically engineered viral particles to deliver the gene to target cell, and non-viral gene therapies employ gene delivery systems comprised of synthetic or semi-synthetic gene formulations. The limitations of viral therapies relate to the residual viral elements within the viral vectors which can be immunogenic , cytopathic, or recombinogenic.

Antisense technology has introduced the possibility of down-regulating or specifically turning off the expression of individual genes. This technology has enormous therapeutic potential. Antisense oligodeoxynucleotides (AON or ODN) constitute sequences of 15 to 21 nucleotides with the order of the nucleotides providing the molecule with the specificity to target genetic material. An oligonucleotide whose bases are tailored to complement part of a particular mRNA, can bind to and complex with that section of the mRNA. This can prevent gene expression which may prevent protein synthesis by passive or reactive inhibition of mRNA translation. Antisense ODN's to DNA seem to inhibit DNA transcription by formation of a triple helix.

Antisense oligonucleotides enter cells by pinocytosis and/or receptor-mediated endocytosis after binding to cell surface antigens. Uncharged oligomers enter cells by passive diffusion and charged oligomers enter by endocytosis. It seems that oligomers are not internalized by cells very efficiently. Methods for improving cellular uptake and biological efficacy of ODN's have been devised, including their conjugation to a synthetic polypeptide poly(L-lysine) tail with or without transferrin, or encapsulation in cationic or antibody - targeted liposomes.

As with other modes of contemporary gene therapy, delivery remains a central and crucial issue. For example, Antisense oligonucleotides per se are thought not to cross the intact Blood Brain Barrier (BBB). There are no studies analyzing the passage of antisense oligomers across the Blood Brain Barrier. Attempts to deliver them across the BBB by hyperosmotic BBB disruption after conjugation or by incorporation into liposomes have, as a whole, been unsuccessful. Direct injection of free antisense results in their rapid breakdown.

Although, most research in vivo gene therapy has focused on the use of recombinant virus vectors, progress has been made toward developing non-viral formulations of gene for in vivo human gene therapy. The advantages of non-viral vectors are that they can introduce DNA into non-dividing cells, do not integrate into the chromosome, do not posses infective risk, and are potentially less expansive than viral vectors. The principle underlying non-viral gene delivery is that the problem of delivering DNA in vivo is not significantly different from the problem of delivering conventional drugs or biological products to intracellular compartment in the body. Non-viral gene therapies involve known drug delivery methods for the administration and targeting of genes to selected cells in vivo, where they express therapeutic products.

Various methods have been described for non-viral gene therapy, ranging from the direct administration of "naked" plasmid DNA to the systemic administration of complex synthetic formulations. Some approaches are aimed at developing "artificial viruses" that attempt to mimic the process of viral infection using synthetic or semi-synthetic components. Others apply the theory and method of advanced, particulate drug delivery to administer DNA to selected somatic targets. These approaches employ plasmid DNA complexes containing lipids, proteins, peptides, or polymeric carriers. The principle disadvantage associated with non-viral systems has been insufficient levels of gene expression, irreproducibility and significant variations in gene expression on various cell types.

The two classes of synthetic gene delivery systems that have been investigated most actively involve the use of either cationic liposomes or polycationic polymers. The assembly of these systems is achieved by an electrostatic condensation of the "anionic" DNA with the "cationic" moiety of either a lipid or a synthetic polymer. The cationic polymer-based systems have been most widely associated with the formulation of receptor-mediated gene delivery systems. This technique employs the ability of receptors on the surface of a variety of different cells to efficiently bind and internalize a ligand. Several ligands have been exploited for the efficient internalization of DNA-ligands complexes. These include: asialoorosmucoid and other galactosylated proteins which target the hepatic asialoglycoprotein receptor; transferrin which binds to the transferrin receptor and mannosyl which is recognized by the mannose receptor of macrophages. Targeting ligands are covalently linked to a polycation polymer, typically to poly(lysine) derivatives, and then form a ligand-poly(lysine)-DNA complex by the ionic interaction between the positively charged poly(lysine) and the negatively

charged DNA. Often, an endosomolytic agent is added to the transfection mixture to induce endosomal lysis and enhance DNA release from the endosome in order to achieve high transfection efficiency. The efficiency of poly(lysine)-DNA conjugates in transfecting numerous cell types in vitro has been demonstrated, but their potential usefulness for in vivo human gene therapy is limited due to their cytotoxicity.

More advanced polymeric gene delivery systems employ macromolecules with a very high cationic charge density that act as an endosomal buffering system, thus suppressing the endosomal enzymes activity and protecting the DNA from degradation. The high cationic charge density mediates both DNA condensing and buffering capacity, that diminish the requirement for an endosomolytic agent addition.

Polymers used in gene transfer

The polycations used for gene complexation are polyamines that become cationic at physiologic conditions All polymers contain either primary, secondary, tertiary or quaternary amino groups capable of forming electrostatic complexes with DNA under physiologic conditions. The highest transfection activity is obtained at a 1.1 -1.5 ratio of polycation to DNA. The most studied polyamines for gene transfer includes, poly(lysine) and its derivatives, polyamidoamine starburst dendrimers, polyethyleneimine, natural and modified polysaccharides, and acrylic cationic polymers. The details for each polymer class are described in Domb et al. (A. Domb, M. Levy, Polymers in gene therapy, Frontiers in Biological Polymer Applications, R.M. Ottenbrite (ed), Technomic, Vol. 2, 1999,1-16.).

Polycations may be more versatile for use than the liposomes and other conventionally used spherical gene carriers. Several polycations have been reported to induce gene expression for example diethylaminoethyl dextran and other cationized polysaccharides [F.D. Ledley, Huiman Gene Therapy, 6, 1129, 1995; Yamaoka et al. Chemistry Letters, 1171-72, 1998]. These polymers have little structural similarity with each other except possessing cationic groups.

Cationic polysaccharides have been used for gene delivery. Chitosan, a linear cationic polysaccharide was suggested by several authors for gene delivery [K.W. Leong et al, DNA-Chitosan nanospheres: Transfection efficiency and cellular uptake, Proceed. Intl. Symp. Control. Rel. Bioact. Mater. 24:75-76, 651-652, 671-674, 1997; R. Richardson, H.V.J. Kolbe, R. Buncan, Evaluation of highly purified chitosan as a potential gene delivery vector, Proceed. Intl. Symp. Control. Rel. Bioact. Mater. 24:649-650, 1997] DNA-chitosan nanospheres were found to be significantly less toxic than poly(L-lysine) or Lipofectin using the MTT test. Compared to standard Lipofectamine mediated gene transfer, these nanospheres yield lower levels of gene expression in HEK 293 (human embryonic kidney cells), IB3 (bronchial epithelial cells) and HTE (human tracheal epithelial cells). Surface modification of DNA/chitosan complex nanoparticles by covalently binding poly(ethylene glycol), transferrin and mannose-6-phosphate receptor to facilitate entry into cells and improve storage stability was also studied. The Purified and hydrophobized chitosan has also been suggested as carrier for genes [K.Y. Lee, I.C. Kwon, Y.H. Kim, W.H. Jo, S.Y. Jeong, Selfaggregates of hydrophobically modified chitosan for DNA delivery, Proceed. Intl. Symp. Control. Rel. Bioact. Mater. 24:651-652, 1997].

Midox (WO 95/30020) describes a polypeptide such as polylysine modified at the g-amino group with a molecule bearing hydroxyl groups. Genzyme describes in WO 97/462 lipid derivatives of short chain alkylamines such as spermine and spermidine for use in gene transfection. For example one or two spermine or spermidine groups attached to cholesterol via an amide or carbamate bonds. WO 98/27209 to Emory Univ. describe a range of modified cationic polypeptides based on lysine for use in gene transfection.

The polymers described in the prior art can be grouped into two catagories: One including linear or dendrimeric polymers with random distribution of amino groups which are part of the polymer backbone such as poly(ethylene imines), poly(amido-amine) dendrimers, and poly(alkylamino-glucaramide). The second including linear polymers with a single primary secondary or tertiary amino group attached to the polymer units. Examples of such polymers are: poly(dimethylaminoethyl methacrylates), dimethylamino dextran, and polylysines.

All of the above polymers are polycations with a random distribution of the cationic sites. This randomness is probably the reason for the fact that these polymers may work for some nucleotides and cell types and not for others. Most of these polymers are toxic to cells, and non-biodegradable, while the polymers based on amino acids such as polylysines are immunogenic.

It can be said that in the prior art, little attention was given to:

1. the structure of the polycation, the charge density and space distribution of cationic groups in the polymer to optimize complexation with anionic nucleotides;

2. the type of cationic groups, primary, secondary or tertiary groups were considered as cationic sites.

3. the toxicity and immunogenicity of the polymer;

4. the biodegradability and elimination properties of the polymer carrier;

In general, it has been believed that the cationic charge of the polymers is the main factor important for complexation and transfection. Also, these cationic polymers did not result in high enough transfection yield for commercial interest in ex-vivo experiments, in addition to animal experimentation.. The degradation and elimination of the polymer carrier was not carefully treated and most polycations described for use in gene therapy are not biodegradable and/or toxic.

In designing a universal polycation system for gene delivery one should consider the way in which a plasmid becomes active in the cell and tissue. The plasmid has first to be protected from DNA degrading enzymes in the extracellular medium, then penetrate the cell wall, protected from degrading systems, i.e. the lisosome and enzymes, in the intracellular medium until it is internalized in the nucleus, penetrate into the nucleus and being released in its active form from the polymer carrier.

This invention describes a versatile and universal polycation system based on oligoamine grafted on natural or synthetic polysaccharides that is capable of complexing various plasmids and antisense, administering them into various cells in high yields and into the nucleus in active form to produce the desired protein.

It is the objective of the present invention to provide polycations that:

1. better fit the complexation requirements for effective delivery of a plasmid or an antisense;

2. biodegrade into non-toxic fragments at a controlled rate;
3. non-toxic and no-immunogenic in vivo;
4. form a stable enough complex with low and high molecular weight polynucleotides including therapeutic plasmids and antisense.
5. provide effective polymeric delivery system that result in a high transfection yield in a range of cells and in tissues.
6. can be reproducibly prepared at an affordable cost.

Another objective of this invention is to provide a controlled release of DNA in tissue or cell by complexing DNA with designed polymers that gradually de-complex and release the DNA or by incorporation of the complexed polynucleotides in a biodegradable matrix which will release the DNA in the site of insertion for periods of weeks and months.

Thus, according to the present invention there is provided a biodegradable polycation composition for delivery an anionic macromolecule, comprising:

a) a polysaccharide chain having an amount of saccharide units ranging from 2 to 2000;

b) at least one grafted oligoamine per 5 saccharide units, wherein said oligoamine is selected from the group consisting of a linear, branched and cyclic alkyl amine having at least four amino groups with at least one being a primary amine; and

b) at least one amphiphilic residue.

In a preferred embodiment of the present invention said anionic macromolecule is selected from the group consisting of a plasmid, oligonucleotide, antisense, peptide, protein and combinations thereof.

In another preferred embodiment of the present invention said polysaccharide chain is selected from the group consisting of dextrans, arabinogalactan, pollulan, cellulose, cellobios, inulin, chitosan, alginates and hyaluronic acid.

In a further preferred embodiment of the present invention said saccharide units are connected by a bond selected from the group consisting of acetal, hemiacetal, ketal, orthoester, amide, ester, carbonate and carbamate.

In an even further preferred embodiment of the present invention said polysaccharide is a synthetic polysaccharide formed from the condensation of an aldaric acid and a diaminoalkane.

In a preferred embodiment of the present invention said grafted oligoamine is grafted to said polysaccharide chain by a bond selected from the group consisting of amine, amide and carbamate. In another preferred embodiment the oligoamine has the formula:

$$NH_2\text{-}[CH_2)_x\text{-}N\text{-}(R)\text{-}CH_2)_y\text{-}N\text{-}(R')\text{-}(CH_2)_z\text{-}]_n\text{-}NH_2$$

wherein x, y, z are an integer between 0 and 4 and x+y+z+ is between 1 and 4 and n is at least 1 when x+y+z=2 or more, or at least 2 when x+y+z=1 and wherein R and R' groups are H or an aliphatic side group of 1 to 6 carbons.

In a further preferred embodiment of the present invention said oligoamine is selected from the group consisting of spermine and derivatives thereof.

In an even further preferred embodiment of the present invention said oligoamine is selected from the group consisting of a linear and branched ethyleneimine oligomer having up to 10 ethylene imine units.

In a preferred embodiment of the present invention said amphiphilic residue is selected from the group consisting of fatty chains, phospholipids, cholesterol derivatives, ethylene glycol oligomers and propylene glycol oligomers, wherein said ethylene and propylene glycol oligomers have a fatty chain block on one side.

In a further preferred embodiment of the present invention said amphiphilic residue is connected to said polysaccharide chain by a bond selected from the group consisting of an amine, amide, imine, ester, ether, urea, carbamate and carbonate.

In an even further preferred embodiment of the present invention said amphiphilic residue facilitates the crossing of the polycation through biological membranes.

In a preferred embodiment of the present invention said polycation composition is not toxic or immunogenic.

In an even further preferred embodiment, the composition of the invention further comprises a ligand for facilitating the binding of said composition to a predetermined type of cell or tissue.

It is a further an objective of the invention to provide a pharmaceutical composition comprising the composition described above, in combination with a pharmaceutically acceptable carrier.

The present invention describes a range of biodegradable polycations based on grafted oligoamine residues on synthetic or a natural polysaccharides which are effective in delivering plasmids and antisense for a high biological effect. The grafting concept where side chain oligomers are attached to either a linear or branched hydrophilic polysaccharide backbone, allows two/three dimensional interaction with an anionic surface area typical to the double or single strand DNA chain. This type of flexible cationic area coverage is not available with non-grafted polycations or low molecular weight cations. Low molecular weight amines and their lipid derivatives such as the lipofectin and lipofectamine have a localized effect on the DNA which the degree of complexation is dependent on how these small molecules organized around the anionic DNA. Each molecule has to be synchronized with the other molecules at all times of the transferction process whereas when the oligoamines are grafted on a polymer they are already synchronized and each side chain helps the other side chains to be arranged to fit the anionic surface of the given DNA. By grafting the functional groups is an average distribution along a polymer chain at a certain distance between each other (for example, grafting an oligoamine chain every one, two, three or four polymer unit may provide optimal complexation with various DNAs.

The use of biodegradable cationic polyol carriers is especially suitable for transfection and biological applications because they are water soluble and miscible in aqueous vehicles. The resultant grafted polymers are water soluble or dispersible in water, it can

be readily transported to cells *in vivo* by known biological processes, and acts as an effective vehicle for transporting agents complexed with it.

The compositions of the present invention are composed of a natural or synthetic polysaccharide backbone with a grafted complexation functionality, i.e. aliphatic organic cationic residues containing at least 4 amino groups, wherein at least one of them is a primary amino group. The alkyl amino cationic residues are distributed in an optimal charge distribution tailored for as many plasmid or oligonucleotide for optimal transfection results. The polymer has hydrophobic/hydrophilic side groups that allow penetration of the polymer-plasmid complex into cells for transfection.

Detailed Description of the Invention:

The present invention provides a new class of non-viral polymeric vectors that can be used for both *in vitro* and *in vivo* transfer of biologically active molecules. In particular, these vectors can be used for gene transfer applications. The polycationic compositions of the present invention can achieve gene transfer efficiencies in vitro that are superior to commercially available cationic liposome preparations. Further, the low toxicity and lack of serum inhibition of the compositions is suitable for *in vivo* use. The present invention provides a vector that can achieve *in vivo* gene transfer efficiencies that compare favorably to viral vector systems. The present invention further provides a method to increase the capacity of solutions to carry complexes of nucleic acids and the polymeric vector without precipitation or toxic ionic effects on cells.

Furthermore, the unique polycationic structure of this class of polymers associates with many suitable bioactive molecules, including proteins and other

compounds that possess multiple anionic sites. The polymer can act as a carrier to deliver the associated bioactive molecule, *in vivo* or *in vitro*, to the cells of interest for the bioactive molecule.

In addition, the unique polycationic structure of this class of polymers are biodegradable and readily eliminated from the body after administration.

In one aspect the invention provides a complex comprising a nucleic acid and a transfection agent, wherein:

a) the transfection agent is obtained by the conjugation of short aliphatic oligoamine to a polysaccharide containing hydrophobic and/or amphiphilic side groups to allow penetration into cells.

b) the short aliphatic oligoamine conjugated to the polymer contains at least one primary amine in addition to at least three primary, secondary, tertiary or quaternary amines.

c) The hydrophobic and/or amphiphilic sites attached to the polymer are for example fatty chains, phospholipids or cholesterol derivatives or ethylene or propylene glycol oligomers with or without a fatty residue block, which have the capacity to allow penetration into cells. The density and quality of the hydrophobic side groups are selected to allow optimal transfection both in vitro (cells) and in vivo (humans).

d) The transfection agent is able to deliver a gene, antisense or nucleic acids into cells and/or nucleus, release them in active from to allow substantial biological effect by the gene or antisense and, biodegrade into non-toxic fragments that are eliminated from the cell or the body.

As used herein the term "transfection agent" means any chemical agent capable of facilitating the entry of a nucleic acid into a eukaryotic cell.

As used herein the term "nucleic acid" means a polymer of nucleotides, and specifically includes plasmids, coding DNA sequences, mRNAs, and antisense RNA molecules. A nucleic acid can be single – or double – stranded. The nucleic acids can also contain one or more substitute linkages. These substitute linkages include conventional alternative linkages such as phosphorothioate and phosphoramidate, and are synthesized as described in generally available literature. Nucleic acids also include those nucleotides in which the sugar moiety has been modified by, for example, substitution of one or more hydroxyl groups with halogen, aliphatic groups, or functionalized as ethers, amines, or wherein the ribose or deoxyribose is replaced with other functionally equivalent structures. In particular, the sugar-phosphate backbone may be replaced with a non-carbohydrate backbone such as a peptide or other type of polymer.

As used herein the term "primary amine" means any amine that possesses one or more primary amine functionality.

As used herein the term "secondary amine" includes amine moieties having at least two pendent hydrocarbon groupings, and also includes, in the appropriate context, tertiary and quaternary amines.

As used herein "a" can mean one or more, depending upon the context in which it is used.

As used herein "aliphatic" and "aromatic hydrocarbons" include both substituted and unsubstituted compounds, wherein the substitution can occur in the backbone or

pendent groupings of the hydrocarbon. Aliphatic compounds may be branched or straight chained.

As used herein "polysaccharide" means, linear, branched or crosslinked natural or chemically modified polysaccharides. It also includes synthetic copolymers having at least 40% saccharide units in the polymer backbone. A particular example is polyamides of glucaric acid with alkanediamines.

As used here "oligoamine" means a linear, cyclic and branched alkaneamine that contain at least four amino groups with at least one of them in a primary amine which will remain primary after grafting. The molecular weight of the oligoamine is limited to about 2,000 Daltons.

The present invention relates to a novel class of polycationic polysaccharides having effective nucleic acid transfection properties and bioactive agent delivery attributes. The polymers are obtained from the conjugation of an oligoamine to a polysaccharide chain. The polysaccharide carrier, the oligoamine and the grafting ratio bond type are selected to enhance the degree and efficiency of transfection. For example, polymers can be selected based upon the density and distribution of the cationic sites on the polymer to obtain transfection agents that are tailored to the anionic charge distribution of the nucleic acid being transfected, and the anionic charge distribution of the type cell being targeted. Various substituents can also be incorporated into the polymer to affect the properties of the polymer by improving the transfection efficiency thereof.

The present invention provides (1) a class of polycationic polymers, (2) a class of complexes comprising these polymers with nucleic acids, and (3) a class of complexes

comprising these polymers with suitable anionically charged bioactive agents. The class of polycationic polymers comprises products obtained by the grafting of an oligoalkaneamine onto a suitable polysaccharide, wherein the grafted oligoamine contains at least one primary amine and at least three more amines.

By "at least one" primary amine and "at least three secondary amines" is meant that the primary amine has minimally one amine, but can preferably also have two, three, four, five, six, or seven of each amine. In a particularly preferred embodiment, the grafted primary amine has one primary amine and three secondary amines.

Examples of suitable polysaccharides include, for example, dextrans, arabinogalactan, pollulan, cellulose, chitosan, inuline, hyaluronic acid, and alginates having from 2 to 2,000 saccharide units. Other classes of polysaccharides are polyureas or polyamides of aldaric acids such as mucic acid, glucaric acid, galactaric acid, xylaric acid, and their various isomers polycondenced with aliphatic diamines. The copolymerization of the comonomers may be performed generally by methods known in the art, including by condensation reactions. Examples of suitable polycondensation techniques are described in detail in Kieley et al., J. American Chemical Society, 116, 571-578 (1994), Kieley et al., U.S. Pat. Nos. 3,225,012; 5,434,233; 5,312,967; 5,473,035; 5,833,230; and 5,329,044; and Dewar et al.

The ionic association of the polymer/nucleic acid charges neutralizes the anionic charges on the nucleic acid and allows the complex to interact and bind more favorably with the negatively charged cell surface. If an excess of cationic sites are present on the polymer, i.e. more than are necessary to neutralize the anionic charges on the nucleic acids, these excess cationic charges may facilitate the attraction of the complex

to the ionically charged surface of the cell, thereby facilitating entry of the complex into the cell. The polymers may also compact the nucleic acids upon complexation, which further enhances the likelihood of entry.

Although the present invention is limited to grafted oligoalkylamines, various substitutents can be incorporated into the polymer carrier. For example, the hydroxyl groups on the aliphatic chain of the monosaccharides can be substituted with aliphatic hydrocarbons, amides, azo, carbamate, carboxylic esters, ethers, thioethers, thiols, fluorescent derivatives, and sulfonic acids. One is often able to increase the hydrophobicity of the polymer (where hydrophobicity is desired) by alkylating the secondary amines with long chain hydrocarbons. Alternatively, one may increase the amphiphilicity by attaching a polyethylene glycol (PEG) chain.

The structure of the polymer can also be altered, by known techniques, to optimize the transfection and delivery efficiency of the polymer for each cellular target on the basis of the physiological and biological characteristics of that target. For example, the efficiency of gene delivery to cells can be enhanced by the addition of peptides with the nuclear targeting signal of simian virus 40 to the polymer. Several protein ligands are also known that can be covalently coupled to the polymer and then incorporated into a ligand-nucleic acid complex. The resulting complexes retain their ability to interact specifically with cognate receptors on the target cell.

Another method for improving the efficiency of gene delivery is to enhance the release of DNA from the endosome after it has entered the cell. Adenoviral particles can be coupled to the polymer to increase this efficiency. Synthetic peptides can also be designed and incorporated into the polymer in order to enhance endosomal release.

The biodegradable polycation compositions of the present invention are of general use for gene transfer and bioactive agent delivery with respect both to cell type and size of nucleic acid or bioactive agent because the transfection is driven by ionic interactions. Any selected cell into which transfection of a nucleic acid or delivery of a bioactive agent (via transfection or other means) would be useful can be targeted by this method, by administering the composition in a suitable manner to bring the complosition into contact with the selected cell, as is known in the art. Cells can be within a tissue or organ, for example, supplied by a blood vessel into which the composition is administered. The composition of the present invention can be formulated into a slab, pellet, microsphere and nanosphere made of a biodegradable component such as a biodegradable polymer or fat to allow targeting and/or controlling long term release of the gene complex to the blood system or to a specific site as known in the literature for common bioactive molecules. Alternatively, for example, the composition can be directly injected into the target tissue or organ. As a further example, the lungs can be targeted by inhalation or intratracheal injection of the complex or particles containing the complex. The invention has application to all eukaryotic cells; it can be used particularly for mammalian cells and subjects, such as humans, cows, horses, sheep, pigs, rats and mice. Some examples of cells that can be targeted by the composition of the present invention include fibroblasts, epithelial cells, endothelial cells, blood cells and tumor cells.

Due to the fact that the polyol backbone according to one of the embodiments of the invention is both biodegradable and regularly imported into living cells as part of normal biosynthetic processes, it is nontoxic and nonimmunogenic, which offers a distinct advantage over viral vectors when used as transfection agents. Similarly

because polyols do not generally contain natural binding sites for serum, the polyol backbone is not negatively impacted by circulating serum proteins such as heparin and albumin. Complexes formed with the polymers thus reach targeted cells intact without significant serum inhibition, in contrast to polycationic lipids which are substantially impacted by natural systemic serums.

The amount of DNA that is carried in solution can also influence the degree of transfection of the composition. The concentration of DNA in solution is often limited by its tendency to precipitate at higher concentrations. In some applications, the DNA concentration in solution is limited to about 1.0 g/l. Increased amounts of DNA-polymer in solution which does not precipitate, may be achieved if proper methods of preparation and optimal polymer to DNA ratio and polymer structure are used. In such preparations it may be possible to obtain solutions carrying 20 grams of DNA per litter of solution.

Suitable delivery and transfection conditions are when the cell and composition temperature is between about 18°C and about 42°C, with a preferred temperature being between about 22°C and about 37°C. For administration to a cell in a subject, the complex, once in the subject, will of course adjust to the subject's body temperature. For *ex vivo* administration, the complex can be administered by any standard method that would maintain viability of the cells, such as by adding the complex to a culture medium (appropriate for the target cells) and adding this medium directly to the cells. The medium used in this method should be aqueous and non-toxic so as not to render the cells non-viable. In addition, the medium can contain nutrients for maintaining viability of cells, if desired.

The composition can be administered *in vivo* by parenteral administration, e.g., by intravenous injection including regional perfusion through a blood vessel supplying the tissue(s) or organ(s) having the target cell(s). Injectables can be prepared in conventional forms, such as liquid solutions, suspensions, or emulsions. A slow release or sustained release system can also be used, allowing the maintenance of a constant dosage level.

Other means of administration can include inhalation of an aerosol, subcutaneous, intraperitoneal or intramuscular injection, topical administration such as to skin wounds and lesions, direct transfection into, e.g., bone marrow cells prepared for transplantation and subsequent transplantation into the subject, and direct transfection into an organ that is subsequently transplanted into the subject. Further administration methods can include oral administration, particularly when the complex is encapsulated, or rectal administration, particularly when the complex is in suppository form.

A pharmaceutical composition according to the present invention can include the composition and a pharmaceutically acceptable carrier suitable for the selected mode of administration. A pharmaceutically acceptable carrier includes any material that will not cause any undesirable biological effects or interact in a deleterious manner with the biological host or with the components within the pharmaceutical composition. A pharmaceutical composition can further include other medicinal agents, pharmaceutical agents, adjuvant, diluents, stabilizers, etc., as long as they do not interfere with the action of the composition. Actual methods of preparing such dosage forms are known or will be apparent to those skilled in the art, (for example - Martin, E.W. *Remington's Pharmaceutical Sciences,* latest edition, Mack Publishing Co., Easton, PA.)

While the invention will now be described in connection with certain preferred embodiments in the following examples so that aspects thereof may be more fully understood and appreciated, it is not intended to limit the invention to these particular embodiments. On the contrary, it is intended to cover all alternatives, modifications and equivalents as may be included within the scope of the invention as defined by the appended claims. Thus, the following examples which include preferred embodiments will serve to illustrate the practice of this invention, it being understood that the particulars shown are by way of example and for purposes of illustrative discussion of preferred embodiments of the present invention only and are presented in the cause of providing what is believed to be the most useful and readily understood description of formulation procedures as well as of the principles and conceptual aspects of the invention.

With specific reference now to the figures in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

Example 1: Grafting of oligoamine on polysaccharides and the biological testing thereof.

A range of polyethyleneimine (PEI, MW=600), spermine (4 amines) and spermidine (3 amines) conjugated to arabinogalactan (AG, a branched polysaccharide, MW=25,000) dextran (Dex, a linear 1,6-polyglucose, MW=30,000) or pollulan (Pol, a linear 1,4 polyglucose, MW=50,000) were prepared. The oligoamines were grafted by an amine or imine bond after oxidation of the polysaccharide into a polyaldehyde. The difference between the polymers tested for biological activity were:

1. the oligoamine used, either PEI, spermine or spermidine;
2. the type of polysaccharide, AG, pollulan or Dex;
3. the type of bond, amine or imine;
4. the content of oligoamine per saccharide unit;

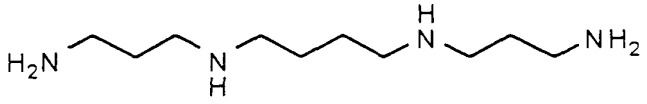
All polymers contained a Triton 100 residue grafted on about 4% of the saccharide units in the polymer carrier. This grafting was applied to the polysaccharides by reacting an epoxide or carboxylic acid derivative of Triton with the polysaccharide in a mixture of DMF:water as described below and in J. Pitha et al. (Detergents linked to polysaccharides: preparation and effect on membranes and cells, Eur. J. Biochem. 94, 11-18, 1979).

The following materials were prepared and used in the synthesis of the various grafts:

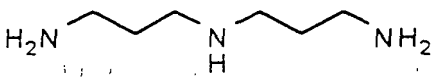
- AG (1:1): oxidized Arabinogalactan produced by reacting 1 mole of saccharides units and 1 mole of periodate (35% of saccharides units were converted to di-aldehydes).

- AG (1:5): oxidized Arabinogalactan produced by reacting 1 mole of saccharides units and 0.2 mole of periodate (8% of saccharides were converted to di-aldehydes).
- Dex (1:1): oxidized Dextran produced by reacting 1 mole of saccharides and 1 mole of periodate (50% of saccharide units were converted to di-aldehydes).
- Pol (1:1) : oxidized pollulan produced by reacting 1 mole of saccharides units and 1 mole of periodate (degree of oxidation was not determined).
- PEI: Polyethylene imine (Mw=600).
- Red: Reduced conjugates (amine bonds).
- Unred: Unreduced conjugates (imine bonds).
- Triton-X100 is an amphiphilic molecule of the structure:
  C_8H_{17}-C_6H_4-$(O\text{-}CH_2\text{-}CH_2)_{9\text{-}10}$-OH

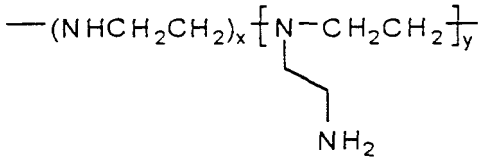
Structures of amino-compounds used for conjugations



Spermine



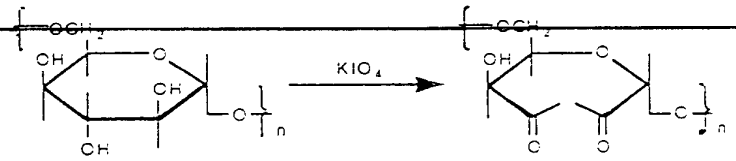
Spermidine



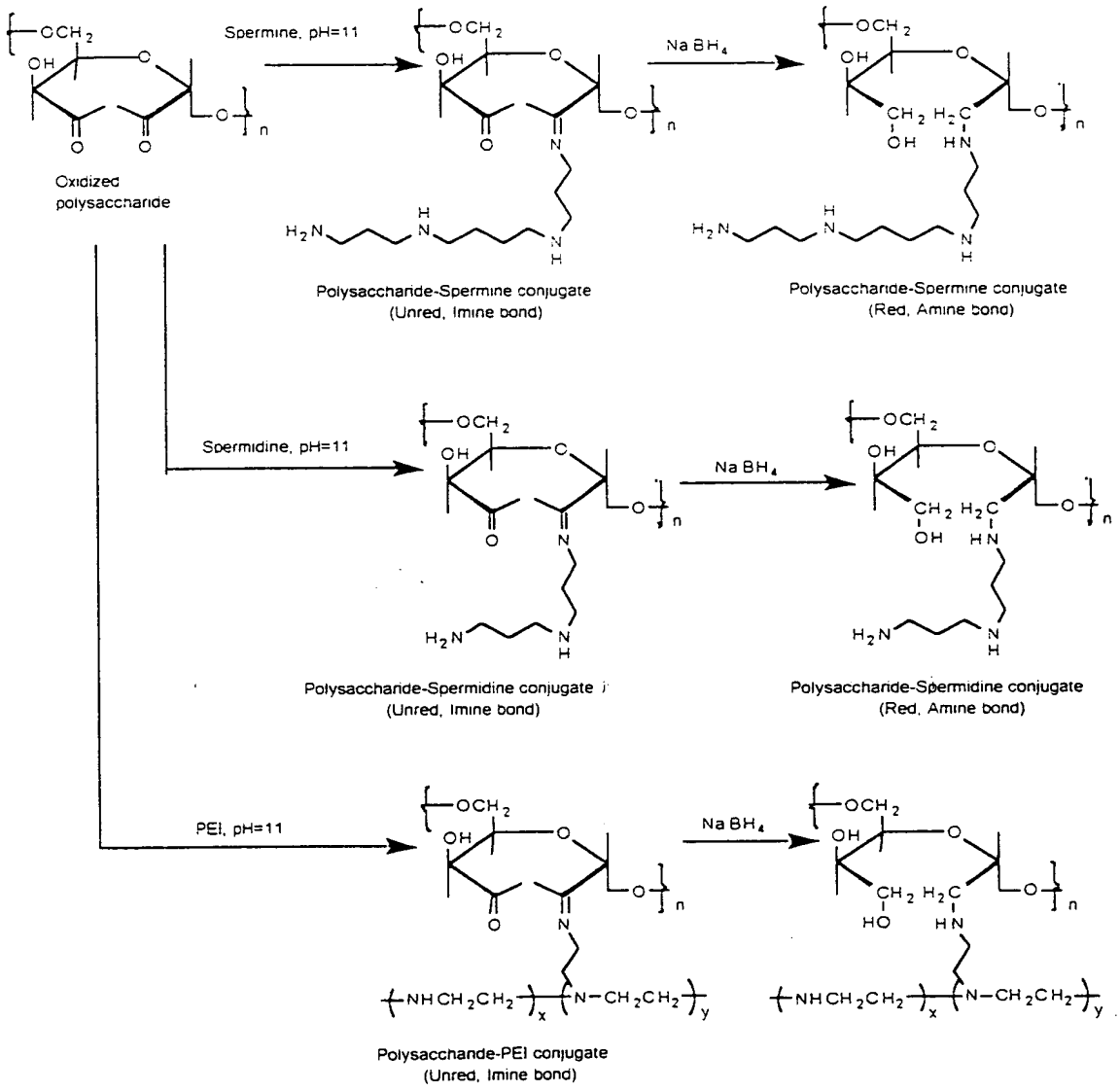
Polyethylene imine (Mw=600)

Oxidation of Polysaccharides (I) and typical reactions between oxidized polysaccharides and polyamines (II) are presented by the following formulas:.

(I)


KIO4

(II)


Spermine, pH=11
NaBH4
Oxidized polysaccharide
Polysaccharide-Spermine conjugate (Unred, Imine bond)
Polysaccharide-Spermine conjugate (Red, Amine bond)
Spermidine, pH=11
NaBH4
Polysaccharide-Spermidine conjugate (Unred, Imine bond)
Polysaccharide-Spermidine conjugate (Red, Amine bond)
PEI, pH=11
NaBH4
Polysacchande-PEI conjugate (Unred, Imine bond)

Grafting of hydrophobic or amphiphilic residues:

The attachment of hydrophobic or amphiphilic residues can be carried-out by various methods adopted from the literature (for example, Y.Takakura, et al. Control of pharmaceutical properties of soybean tyrosine inhibitor by conjugation with dextran I: synthesis and characterization, J. Pharm. Sci. 78, 117-121, 1989; G.H. Hermanson, Bioconjugate techniques, Academic Press, 1996). The following methods were used by the present inventor:

The hydrophobic residue was generally conjugated by an ester, amide, imine, amine, urethane or carbonate bonds depending on the availability of the functional groups on the conjugated component. For example, fatty acids such as hexanoic acid or oleic acid are bound to hydroxyl or amine groups on the polymer carrier using activated acids such as anhydride or acid chloride derivatives or activating agents such as dicyclohexylcarbodiimide (DCC) and its derivatives that are more suitable for aqueous mediums. Alternatively, hexyl or oleyl alcohols or amines have been conjugated via carbonate or urethane bonds using phosgene derivatives. Polyethylene glycol oligomers and derivatives were conjugated either directly via the hydroxyl end or by converting the hydroxyl end group to a carboxylic acid (by reacting the alcohol with succinic, glutaric or maleic anhydride) or to a reactive epoxide group (by reacting with epichlorhydrine). The grafting reactions are conducted in hydrophilic solutions where the polymer carrier is soluble in or at least dispersed in fine particles with large surface area. Typical mediums are dimethylformamide (DMF), N-methyl pyrrolidone, dimethylsulfoxide (DMSO) and their mixtures with water.

The amount of grafting suitable for cell penetration and transfection is in the range of 1 to 10% of the repeated units building the polymer carrier, i.e. saccharide units. This amount is dependent on the nature of the attached group, the nature of the final product and the polymer carrier.

In a typical experiment Triton-X100 (7g, 10 mmol) was dried by azeotropic distillation with toluene. After solvent evaporation, $SnCl_4$ (5 □l) and epichlorhydrin (1.4g, 15 mmol) and the mixture was kept at 100oC overnight. The solvent was evaporated and mixed with ether and extracted with cold 1N NaOH. The etheric layer was dried over $MgSO_4$ and evaporated to dryness to yield the epoxy terminated Triton (70% yield). The product was identified by TLC (silica, toluene:chloroform) and by H NMR (aromatic protons 5.9-6.7 ppm).

Alternatively, dry Triton-X100 was reacted with succinic anhydride (1:1.1 mole ratio) in toluene at reflux overnight to yield the corresponding succinate derivative as determined by H-NMR, IR and titration.

The chloroformate derivative of Triton was prepared from the reaction of the hydroxyl terminal with diphosgene using known procedures. Grafting of these functionalized Triton was conducted in DMF or DMF:water solutions under the proper conditions. For example, epoxide terminated Triton (0.5g) was reacted with dextran (2g) in 1N NaOH (10 ml) overnight at room temperature. The polymer was purified by dialysis against water and lyophilization. The derivatization rate was about 5% of the saccharide groups.

Carboxylic acid Triton-X100 reacted with arabinogalactan in a mixture of DMF and water using water soluble DCC. Chloroformate Triton was reacted with a suspension of polysaccharides in dry DMF for 3 days at room temperature. Triton was reacted in access relative to the saccharide groups in order to obtain a 5% conjugation.

Similar procedures were applied for the conjugation of methoxy-PEG-OH or Lipo-PEG (a diblock polymer of a fatty acid such as stearyl, oleyl or hexanoyl groups with $(PEG)_{10-100}$).

The conjugation of highly hydrophobic residues such as fatty acids and cholesterol to hydrophilic polysaccharides was conducted in an organic solvent such as DMF or DMSO, the conjugation yield was low (1-5% of saccharide units) but suitable for gene formulation.

Hydrophobic or amphiphilic residues were conjugated to oxidized polysaccharide via an amine or imine bonds. In this cases, the hydroxyl terminal of Triton and PEG derivatives were converted to amino terminals by esterification with glycine or alanine or by replacing the hydroxyl group with an amine using the tosylate/ammonia procedure as described below for the amination of polysaccharides.

The amine terminated PEG derivatives, cholesteryl amine or fatty amines are reacted with oxidized polysaccharides in basic buffer solutions (pH9-11) or mixtures of DMF with water over night, similar to the procedures used for the grafting of spermine. The grafting can be conducted during the conjugation of the oligoamines by adding both the oligoamine and the amphiphilic derivative to the reaction mixture. The imine derivatives

were hydrogenated to the corresponding amine bond using NaBH4 in water for 24 hours at room temperature.

Synthesis of oligoamine graft polymers

1) AG-PEI, via imine and amine bonds (1.25 per 1 unit)

0.5 g. of oxidized Arabinogalactan (1:5, ~ 0.5 mmoles of aldehydes) and 0.18 g. of PEI (0.625 mmol) were dissolved in 20 ml borate buffer (0.1M, pH=11). The solution was mixed at room temperature for 48h. Half of the solution (10 ml) was dialyzed against DDW using 12,000 cut-off cellulose tubing and lyophilized to obtain the imine conjugate which was insoluble in water. The other half was reacted with excess sodium borohydride at room temperature over-night, dialyzed against DDW and lyophilized to obtain the amine conjugate which was soluble in water.

aldehyde / PEI (1:1.25, mole ratio).

2) Dex-PEI, via imine and amine bonds (low PEI content, 1 to 7.5 units)

0.3 g. of oxidized Dextran (1:1, 1.875 mmole of aldehydes) and 0.15 g. of PEI (0.25 mmol) were dissolved in 20 ml borate buffer (0.1M, pH=11) and mixed at room temperature for 48h. The solution was treated as above to yield the pure imine and amine conjugates.

aldehyde / PEI (7.5:1, mole ratio).

3) AG-Spermine, via imine and amine bonds (low oxidation, low spermine content)

0.5 g. of oxidized AG (1:5, 0.375 mmole aldehyde) and 0.08 g. of spermine (0.38 mmole) were dissolved in 20 ml buffer borate (0.1M, pH=11) and mixed at room

temperature for 48h. The solution was treated as above to yield the pure imine and amine conjugates.

aldehyde / Spermine (1:1, mole ratio).

4) Dex-Spermine, via imine and amine bonds

0.25 g. of oxidized Dextran (1:1, 1.56 mmole aldehyde) and 0.1 g. of Spermine (0.5 mmole) were dissolved in 20 ml buffer borate (0.1M, pH=11) and mixed at room temperature for 48h. The solution was treated as above to yield the pure imine and amine conjugates.

aldehyde / Spermine (3:1, mole ratio).

5) Dex-Spermidine, via imine and amine bonds

0.5 g. of oxidized Dextran (1:1, 3.125 mmol aldehyde) and 0.2 g. of Spermidine (1.37 mmole) were dissolved in 20 ml borate buffer (0.1<, pH=11) and mixed at room temperature for 48h. The solution was treated as above to yield the pure imine and amine conjugates.

aldehyde / Spermidine (2:1, mole ratio).

6) Amination of Dextran

1.0 g. of oxidized Dextran (1:1, 6.25 mmole aldehyde) were dissolved in 100 ml concentrated ammonium hydroxide (25% ammonia). The solution was mixed at room temperature for 2 days and excess sodium borohydride were added and the solution was stirred for another 24h under the same conditions. Excess ammonia was evaporated and the polymer solution was dialyzed against DDW and lyophilized to dryness.

7) Dex-Spermine, via imine and amine bonds-obtained by high dilution

0.2 g. of oxidized Dextran (1:1, 1.25 mmole aldehyde) and 0.2 g. of Spermine (1.0 mmole) in 200 ml borate buffer (0.1M, pH=11) and mixed at room temperature for 3 days then excess lysine (0.5 g.) was added to saturate excess aldehydes and the mixture was further mixed under the same conditions for another 24h. The solution was treated as above to yield the pure imine and amine conjugates.

aldehyde / Spermine (1.25:1, mole ratio).

8) Dex-Spermidine, via imine and amine bonds-obtained by high dilution

The same procedure and amounts used in (7) but using Spermidine instead of Spermine.

The imine and amine conjugate obtained were soluble in water.

aldehyde / Spermidine (1:1, mole ratio)

9) AG-PEI, via imine and amine bonds-obtained by high dilution

0.5 g. of oxidized AG (1:1, 1.875 mmole of aldehyde) and 1.2 g. of PEI (2 mmole) were dissolved in 1.0 liter borate buffer (0.1M, pH=11) and mixed at room temperature for 3 days. Excess lysine (1.0 g.) was added to the solution to saturate excess unreacted aldehydes and further mixed under the same conditions for another 24h. The solution was treated as above to yield the pure imine and amine conjugates.

aldehyde / PEI (~ 1:1, mole ratio).

10) Dex-Spermine, via imine and amine bonds (high oxidation and spermine content)

0.5 g. Dextran (1:1, 3.125 mmole aldehyde) and 0.25 g. Spermine (1.25 mmole) were dissolved in 40 ml borate buffer (0.1M, pH=11) and mixed at room temperature for 2 days. The solution was treated as above to yield the pure imine and amine conjugates.

aldehyde / Spermine (2.5:1, mole ratio).

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11) AG-Spermine, via imine and amine bonds

0.5 g. of AG (1:5, 0.375 mmole aldehyde) and 0.25 g. Spermine (1.25 mmole) were dissolved in 40 ml borate buffer (0.1M, pH=11) and mixed at room temperature for 2 days. The solution was treated as above to yield the pure imine and amine conjugates.
aldehyde / Spermine (3.3:1 mole ratio).

12. Pollulan-Spermine, via imine and amine bonds (high oxidation and spermine content)

0.5 g. of oxidized Pollulan (1:1, 3.125 mmole assuming 50% oxidation) and 0.25 g. Spermine (1.25 mmole) were dissolved in 40 ml borate buffer (0.1M, pH=11) and mixed at room temperature for 2 days. The solution was treated as above to yield the pure imine and amine conjugates.
aldehyde / Spermine (2.5:1, mole ratio).

13) Pol-Spermine, via an amine bond

0.25 g. of oxidized pollulan (1:1, 1.56 mmole aldehyde assuming 50% oxidation) and 0.25 g. Spermine (1.25 mmole) were dissolved in 40 ml borate buffer (0.1M, pH=11) and mixed at room temperature for 48 h. Excess borohydride was added and further mixed under the same conditions for another 24h. The solution was treated as above to yield the pure imine and amine conjugates.
aldehyde / Spermine (~ 1:1, mole ratio).

Biological testing, evaluation of transfection properties of the polymers

The delivery of GFP and luciferase reporter gene to the mammalian HEK-293 cell line in culture was used to asses the effectiveness of the polymer-DNA complex as a gene carrier.

Gene transfer efficiency was determined by measuring the expression level of reporters in transfected cells. A quantitative transfection assay is employed to achieve rapid optimization of polymer -DNA complex composition. The use of GFP allows direct visualization of efficiency and optimization of time of expression. Serial dilutions of DNA and polymer are performed separately in 96 well plates and combined to give various formulations of the polymer-DNA complex. HEK-293 cells were used as these cell-lines display different levels of efficiency towards many commercially used transfer agents. Cells seeded in 96-well plates are incubated for several hours with different complex compositions. Luciferase activity is assayed by the production of a light signal in an ELISA reader. A transfection yield of about 30% is considered as maximal yield for this type of cells, obtained by calcium phosphate solution. The results reported in Table 1 hereinafter (0-no transfection, ++++ high transfection) are relative to the maximal yield, 30%.

The expression vectors: plasmids with CMV promoters (for high efficient expression in most cells) or promoters more suitable to obtain regulated levels of products such as the b-actin promoter and the PGK promoters were used. All these sets of clones were used with reporter genes, GFP (gene fluorescence protein) and luciferase. The advantage of both reporters (as opposed to other like CAT or b gal) is the very high

sensitivity of the assay for luciferase and the opportunity to detect expression at a very early stage with GFP expression.

The assay for luciferase was used by the ATP/luciferase kit allow detection at fmole range. The assay for GFP expression rely on visualization under fluorescence microscope and monitoring the level of expression as well the efficacy of transfection.

The two vectors used are:

1. PEGH-N (Clontech): useful for mammalian expression; under PCMV promoter; produce fluorescence x35 intense than wield type GFP.
2. PGL2-vector (Promega): useful for expression under SV40 promoter with luciferase gene.

Luciferase assay was performed as in Promega kits (sensitivity of 10-20 mole). The detection of luciferase was performed by Luminometer using kivvetes or 96 well plates formats. The extremely sensitive measurements were very useful as a detection probe to the in vivo (animal tissue) experiment. The current microplates luminometers allows detection limits of less than 20 amol of ATP and a dynamic range with linearity of 7 decades.

The ability of the polymer to form complexes with plasmid DNA was evaluated by gel retardation assay. Samples of beta-galactosidase plasmid were incubated with different amounts of polymer. Following incubation the samples were electrophoresed through agarose gel and stained with ethidium bromide to visualize shifts in the electrophoretic mobility of the complexed DNA. DSC analysis for gene complexation was used.

The present inventors found that many polymers formed complexes with DNA (Table 1), some polymers showed a significant expression in cells with no signs of toxicity to the cells.

It should be noted that graft polymers that do not contain hydrophobic side chains hardly penetrate cells, therefore, to study their effectiveness as gene carriers, the cells were treated with glycerol that helps penetration of the gene complex into cells. The polymers that do not have the capacity to cross the cell wall are not useful for in vivo transfection as they do not penetrate the cell.

A typical experiment was run as follows: HEK cells and COS cells (25,000 per cm^2 or $5x10^5$ cells per 60 mm culture dish) were plated a day before the experiment. The cells were gently washed with serum-free medium and 0.5 ml serum free medium per dish was added. Plasmid DNA (5 mg in 500ml of serum-free sterile tube) was allowed to sit for one hour. Polymer reagent for complexation were added to the plasmid solution and mixed well and added to the cells (total 1.5 ml for a 60mm plate and allow to incubate for about 4 hours. At the end of the incubation period, the cells were gently overlayed with 4 ml of the complete medium with serum at 37°C. To increase the penetration into cells, a 10% glycerol shock for 0.5 min was used. The cell mixture was allowed to incubate for about 48 to 72 hours (for luciferase and beta-galactosidase). The transfection efficiency was determined by counting the transfected cells having GFP (green fluorescence protein).

Table 1: Chemical Data of the Polycations Used for Transfection

#	Code (conjugate)	aldehyde/ polyamine (mole ratio)	% N (found)	% (w/w) of polycation (found)	Mw (GPC)*	Transfection rate
0	PEI	-------	32%	--------	600	+
1	AG-PEI, imine	1:1.25	6.1	18	ND	+/-
1a	AG-PEI, amine	1:1.25	6.14	18.8	ND	+
2	Dex-PEI, imine	7.5:1	ND	low solubility	ND	NR
2a	Dex-PEI, amine	7.5:1	9.77	30	9,500	+
3	AG-Spermine, amine	1:1	1.84	6.65	8,000	+++
4	Dex-Spermine, imine	3:1	7.54	27.25	6770	0
4a	Dex-Spermine, amine	3:1	7.47	27	7820	+++
6	aminated dextran, amin	------	3.36	0.4 amine for each s unit	10,000	+
7	Dex-Spermine, imine	1.25:1	7.12	25.73	11,000	0
7a	Dex-Spermine, amine	1.25:1	7.35	26.56	10,800	++++
5	Dex-Spermidine, imine	1:1	6.92	23.92	9,600	0
5a	Dex-Spermidine, amine	1:1	6.82	23.57	9,000	0
8	Dex-Spermine, imine	2.5:1	7.72	27.9	11570	0

11	AG-Spermine, amine	1:3.3	1.97	7.11	11000	++++
12	Pol-Spermine, amine	2.5:1	9.33	33.68	6840	++++
12a	Pol-Spermine, imine	2.5:1	8.83	31.88	6390	o
4b	Dex-Spermine, amine	1:1	6.90	24.91	ND	++++
control	Dextran-control	------	0	0	24000	0
control	Arabinogalactan-contro	-------	0	0	19000	0

* Molecular weight were determined by GPC. Detection was made using UV lamp at 454 nm after reacting all the polyamine with FITC probe. Retention time of standards were determined in the refractive-index detector. Dex=dextran, AG=arabinogalactan, Pol=pollulan. The polymer codes are related to the experimental description, samples marked with an *a* such as 2 *a* are the amine derivative of the unreduced derivative. The efficiency of transfection was graded as: 0-no activity; +/- traces of activity; + little activity; ++, +++ and ++++ represent increased activity where ++++ indicate activity close to the Ca phosphate transfection.

The conclusions from the above experiment are:

1. The most active grafts were the spermine bound by an amine bond. The imine bond derivative was inactive due to a loss of one amine group used for the imine bond which leaves the grafted oligoamine with only 3 effective amine groups.

2. The grafted spermidine (containing 3 amines) were inactive either as amine or imine conjugation bond due to the presence of less than 4 amine groups in the oligoamine.

3. Spermine grafts prepared at concentrated solution resulted in some binding of both primary amines leaving low primary amine content which result with low transfection.

4. Grafted polyethylene imine (PEI) was effective but less than spermine.

5. All three polysaccharides, dextran, arabinogalactan and pollulan, were similarly effective as biodegradable carriers.

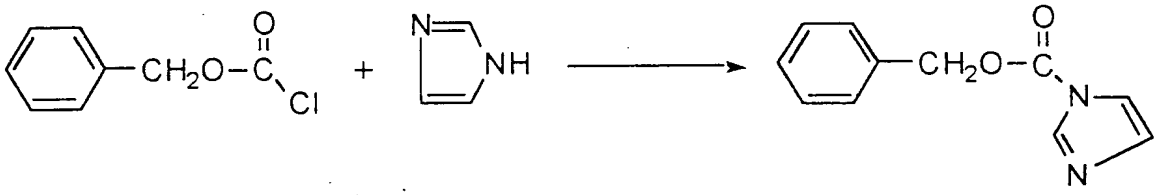
6. The oligoamine grafted polymers without the amphiphilic or hydrophobic side groups showed little penetration capabilities into cells while the polymers having hydrophobic side groups easily penetrated the cells.

Example 2:conjugation of spermine via the secondary amine

In order to graft spermine and similar oligoamines via the secondary amine while leaving the primary amine intact, the following strategy was used. The primary amines were specifically protected by a carbobenzoxy group using a mildly active carbobenzoxy-imidazole. The protected oligoamine was then grafted on dextran or arabinogalactan by reacting the agent with the tosylated or mesylated polysaccharide derivatives. The protecting groups were then removed by a common procedure used for protecting amino acids. The amount of primary amines in the polymer was determined by the fluorescamine or TNBS methods.

Synthesis of N^1-N^{12}-dicarbobenzoxy Spermine

1) Synthesis of Carbobenzoxy imidazole:



Benzyl chloroformate (10.0 g., 58.66 mmol.) in dry DCM was cooled to 0^oC (ice bath), and a solution of imidazole (8.0 g., 117.5 mmol.) in 90.0 ml dry DCM was added dropwise under a nitrogen atmosphere. The reaction mixture was allowed to warm up to room temperature and further stirred for 30 min. The reaction mixture was diluted with DCM and washed 3 times with 10% citric acid (3x100 ml). The organic layer was separated and dried over $MgSO_4$, filtered and evaporated to give a colorless oil. The yield was 90%.

^{1}H-NMR ($CDCl_3$): 5.38 (s, 2H) and 7.00-8.26 (m, 8H) ppm.

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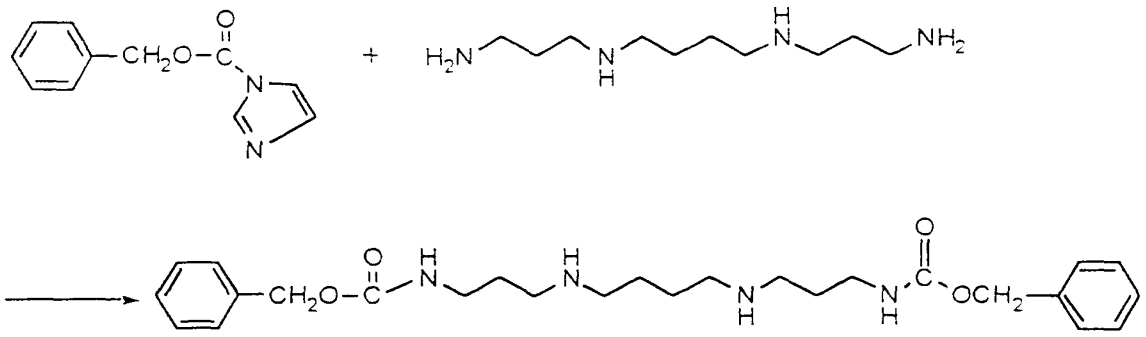
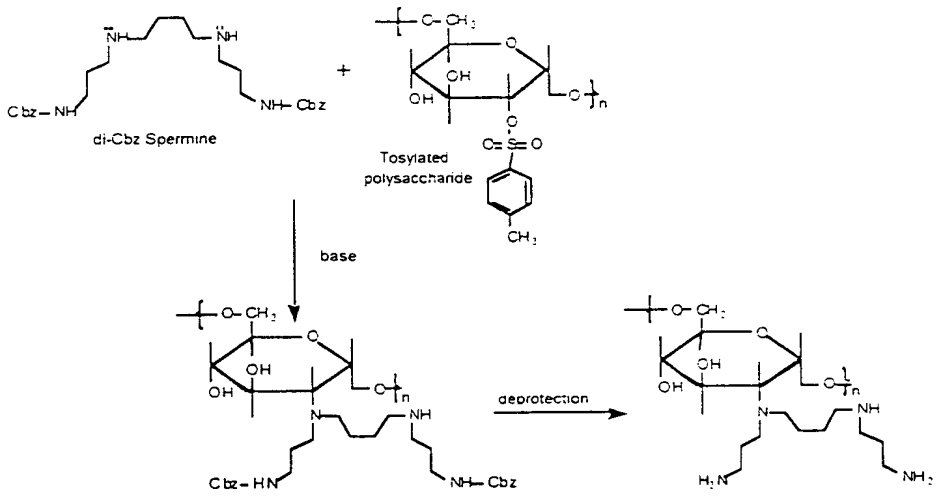
2) Synthesis of N^1-N^{12}-dicarbobenzoxy spermine.



200.0 mg of carbobenzoxy imidazole in 5.0 ml anhydrous DCM and 20.0 mg of DMAP were cooled to 0°C under nitrogen atmosphere. 200.0 mg of spermine was added dropwise in 5.0 ml dry DCM. The solution was stirred for 1 h. at 0°C and over night at room temperature. The solvent was evaporated in vacuum, re-dissolved in 20.0 ml ethyl-acetate and washed twice with 10% citric acid (2x10 ml) and with DDW (2x10 ml). The organic layer was separated and dried over $MgSO_4$, filtered and evaporated to dryness to yield a colorless solid. The solid was suspended in n-hexane, filtered and washed with n-hexane (20 ml) and dried over night in vacuum over P_2O_5. The yield was 60%.

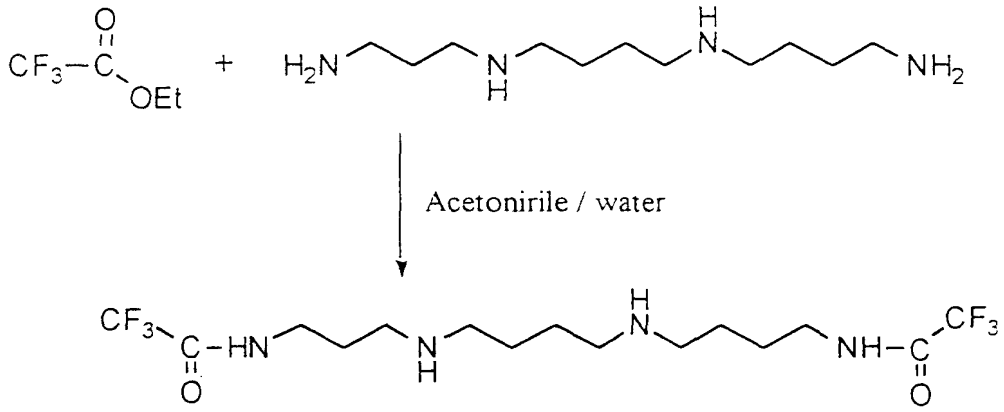
^{1}H-NMR ($CDCl_3$): 1.4-1.7 (m, 20H; hydrogens of spermine), 5.132 (s, 4H; methylene group of benzyl) and 7.2-7.4 (m, 10H; aromatic hydrogen of benzyl group) ppm.

1) Conjugation of N^1-N^{12}-dicarbobenzoxy spermine to Dextran/AG via amine formation:



The tosylated polymer and the protected spermine were dissolved in DMF and mixed under basic conditions (excess triethyl amine) at room temperature for two days to yield the grafted polymer and the protecting groups were removed in HBr in acetic acid.

2) Synthesis of N^{1}, N^{12}-bis(trifluroacetyl) spermine:-



Spermine (5 mmole) in acetonitrile (15 ml) was added ethyl trifluroacetate (25 mmole) and water (12 mmole). The solution was refluxed overnight and the solvent removed to give a buff-colored solid which was washed DCM yielding N^{1}, N^{12}-bis trifluroacetyl spermine as a pale yellow-colored solid in good yield (95%). The solid was recrystallized from ethyl acetate to give a white solid.

The protected spermine was attached to the tosylated polysaccharide as described earlier and the trifluroacetyl group was removed in concentrated ammonia in methanolic solution.

3) Synthesis of mono-(trifluroacetyl) spermine:

Spermine was selectively protected on a primary amino functional group by reaction with triethyl fluroacetate (1.0 eq., MeOH, -78^{o}C for 1h then 0^{o}C over 1h), to afford a mixture containing predominately mono-trifluroacetamide but also di-triflurcacetamide. The mono-protected spermine was purified from the di-protected spermine by column chromatography over silica gel (DCM-MeOH-conc. NH_4OH 70:10:1 to 50:1:1 v/v/v).

The mono-protected amine was then attached to di-aldehyde polysaccharides using a mild basic buffer as a solvent (pH=8.0). The imine conjugates which were obtained were reduced by borohydride and the Tfa protecting group was removed using concentrated ammonia. The conjugate obtained was purified by dialysis using 12,000 cut-off cellulose tubing against DDW and lyophilized to dryness. This method minimized crosslinking and branching between polymer chains and produced a maximum degree of concentration of free amine functionality.

Example 3:

Conjugation of spermine to polysaccharide via amide linkage.

Di-aldehyde polysaccharide was treated with sodium chlorite overnight and purified by anion exchange chromatography (DOWEX-50) to obtain the di-carboxylate form. Carboxylate content was determined by titration and found to be 40% (saccharide units). The polymer was lyophilized to obtain a white solid in good yields.

1.0 eq. of each the anhydrous polymer and spermine were dissolved in dry DMF and a catalytic amounts of DMAP (0.1 eq.) were added. The polymer solution was cooled to $0^{o}C$ and 1.5 eq. of EDC were added. The mixture was stirred overnight under nitrogen atmosphere and DMF was removed in vacuum. The residue was re-dissolved in water and purified by dialysis and lyophilized to dryness. The spermine content attached to the polymer was determined by elemental analysis of nitrogen.

Example 4:

Conjugation of mono-protected spermine to di-carboxylate polysaccharide.

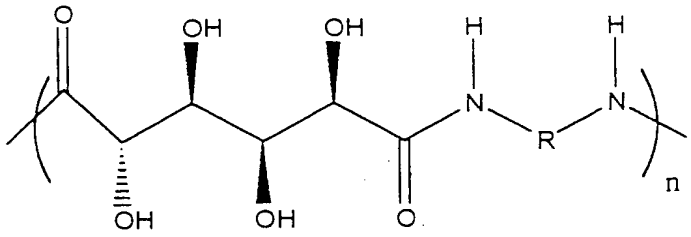
1.0 eq. of each di-carboxylate polysaccharide and mono-trifluroacetamide spermine were dissolved in dry DMF containing a catalytic amount of DMAP (0.1 eq.). The mixture was cooled to $0^{o}C$ and 1.5 equivalents of EDC were added. The mixture was

stirred overnight under nitrogen atmosphere and the DMF was removed under vacuum. Small amount of water were added to destroy excess EDC and the protecting group (trifluro acetate) was removed by treating the solution with concentrated ammonia solution. Excess ammonia was removed in vacuum and the mixture was dialyzed against DDW and lyophilized to dryness. Spermine content was determined by elemental analysis of nitrogen and TNBS method.

Example 5:

Grafting of oligoamine on aldaric acid based polysaccharides:

Spermine and PEI oligoamines were grafted on poly(alkyl-glucaramide using the procedures described in the previous examples. Poly(alkyl-D-glucaramide) of the general structure:



Where R is an alkyl, ethylene glycol oligomer or an alkylamine oligomer;

was obtained from Avanti (Albaster, AL, USA) or synthesized by the procedure described in Keily et al. Hydroxylated nylons based on unprotected esterified D-glucaric acid by simple condensation reaction, J. Amer. Chem. Soc. 116, 571-578, 1994). Grafting of an oligoamine to this polymer was carried-out by the tessellation

method. In a typical experiment, 50 mg of poly(ethylene-D-glucaramide) was dissolved in 4 ml DMF containing 195 mg triethylamine. A solution of tosyl-chloride (122mg) in 2 ml DMF was added to the mixture. The solution was left overnight under argon. DMF is evaporated and the residue is purified by precipitation in ether from a dichloromethane solution. H NMR analysis showed an average of 0.8 tosyl units per monomer unit. This tosylated polymer was reacted with two equivalents of spermine in diluted solution of borate buffer pH9 for three days at room temperature to obtain substitution of the tosyl groups with spermine groups as determined by elemental analysis and TNBS method.

Example 6: Encapsulation of DNA-polycation complex in biodegradable polymers

The Plasmid-polycations of example 3 were encapsulated in a biodegradable microsphere for the purpose of controlled release of the plasmid complex in a specific site in the body. The polymers used for the encapsulation were polymers based on lactide and glycolide and polyanhydrides. Known encapsulation processes such as coaservation and solvent evaporation were used. Methods for encapsulation of bioactive compounds have been described in the literature for example: Nanosphere Delivery Systems, S. Benita, Ed. Marcel Dekker, 1996; Microparticulate Systems for Drug Delivery, H. Berstein and S. Cohen, Eds., Marcel Dekker, 1996.

The plasmid-complex described in Table 1 (7a) above was isolated from the aqueous solution by lyophilization and the powder was used for encapsulation using the solvent evaporation process as follows: to a PLA (MW=2500) solution (100 mg in 0.5 ml dichloromethane), the lyophilized plasmid complex was dispersed using a sonicator. This dispersion was emulsified in about 1 ml 2% polyvinyl alcohol (PVA) in water. The concentrated dispersion was added to 10 ml 0.2% PVA solution and the mixture was mixed vigorously for about five hours at room temperature where microspheres were

formed. The particles were isolated by filtration and washed with water and dried. In vitro release was conducted in phosphate buffer pH7.4.

It will be evident to those skilled in the art that the invention is not limited to the details of the foregoing illustrative examples and that the present invention may be embodied in other specific forms without departing from the essential attributes thereof, and it is therefore desired that the present embodiments and examples be considered in all respects as illustrative and not restrictive, reference being made to the appended claims, rather than to the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

WHAT IS CLAIMED IS:

1. A biodegradable polycation composition for delivery of an anionic macromolecule, comprising:

a) a polysaccharide chain having an amount of saccharide units ranging from 2 to 2000;

b) at least one grafted oligoamine per 5 saccharide units, wherein said oligoamine is selected from the group consisting of a linear, branched and cyclic alkyl amine having at least four amino groups , wherein at least one of said amino groups is a primary amine; and

c) at least one amphiphilic residue.

2. A biodegradable polycation composition according to claim 1, wherein said anionic macromolecule is selected from the group consisting of a plasmid, an oligonucleotide, an antisense, a peptide, a protein and combinations thereof.

3. A biodegradable polycation composition according to claim 1, wherein said polysaccharide chain is selected from the group consisting of dextrans, arabinogalactan, pollulan, cellulose, cellobios, inulin, chitosan, alginates and hyaluronic acid.

4. A biodegradable polycation composition according to claim 1, wherein said saccharide units are connected by a bond selected from the group consisting of acetal, hemiacetal, ketal, orthoester, amide, ester, carbonate and carbamate.

5. A biodegradable polycation composition according to claim 1, wherein said polysaccharide is a synthetic polysaccharide formed from the condensation of an aldaric acid and a diaminoalkane.

6. A biodegradable polycation composition according to claim 1, wherein said grafted oligoamine is grafted to said polysaccharide chain by a bond selected from the group consisting of an amine bond, an amide bond and a carbamate bond.

7. A biodegradable polycation composition according to claim 1, wherein said oligoamine has the formula:

$$NH_2\text{-}[CH_2)_x\text{-}N\text{-}(R)\text{-}CH_2)_y\text{-}N\text{-}(R')\text{-}(CH_2)_z\text{-}]_n\text{-}NH_2$$

wherein x, y, z are an integer between 0 and 4 and x+y+z+ is between 1 and 4 and n is at least 1 when x+y+z=2 or more, or at least 2 when x+y+z=1 and wherein R and R' groups are H or an aliphatic side group of 1 to 6 carbons.

8. A biodegradable polycation composition according to claim 1, wherein said oligoamine is selected from the group consisting of spermine and derivatives thereof.

9. A biodegradable polycation composition according to claim 1, wherein said oligoamine is selected from the group consisting of a linear and branched ethyleneimine oligomer having up to 10 ethylene imine units.

10. A biodegradable polycation composition according to claim 1, wherein said amphiphilic residue is selected from the group consisting of fatty chains, phospholipids, cholesterol derivatives, ethylene glycol oligomers and propylene glycol oligomers.

11. A biodegradable polycation composition according to claim 10, wherein said ethylene and propylene glycol oligomers have a fatty chain block on one side.

12. A biodegradable polycation composition according to claim 1, wherein said amphiphilic residue is connected to said polysaccharide chain by a bond selected from the group consisting of an amine, amide, imine, ester, ether, urea, carbamate and carbonate.

13. A biodegradable polycation composition according to claim 1, wherein said amphiphilic residue facilitates the crossing of the polycation through biological membranes.

14. A biodegradable polycation composition according to claim 1, wherein said polycation composition is not toxic or immunogenic.

15. A biodegradable polycation composition according to claim 1, wherein said composition further comprises a ligand for facilitating the binding of said composition to a predetermined type of cell or tissue.

16. A pharmaceutical composition, comprising the composition of claim 1, in combination with a pharmaceutically acceptable carrier.

For the Applicant
WOLFF, BREGMAN AND GOLLER
by: